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ATR maintains select progenitors during nervous system development

Youngsoo Lee, Erin R.P. Shull, Pierre-Olivier Frappart, Sachin Katyal, Vanesa Enriquez-Rios, Jingfeng Zhao, Helen R. Russell, Eric J. Brown, Peter J. McKinnon

Corresponding author: Peter J. McKinnon, St. Jude Childrens Res. Hosp.

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1st Editorial Decision 30 August 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal, and please excuse the slight delay in its evaluation related to reviewer availability and the vacation season. We have now received the reports of three expert referees (copied below), who all acknowledge the importance of the study as well as its technical quality and presentation. Nevertheless, the referees - in addition to raising a number of specific minor points - all express concerns regarding the amount of conclusive insights into the most significant and unexpected new results from your analysis (especially re. lack of synthetic lethality between ATR and p53/ATM). Should you be able to address these concerns, then we should be happy to consider a revised version further for publication in The EMBO Journal. In this respect, I feel that especially referee 1 provides a number of very constructive experimental suggestions that should help to deepen the understanding of these observations, and to substantiate some of the other major conclusions.

I am therefore inviting you to prepare a revised version of the manuscript, attempting in particular the experiments proposed by referee 1 and making sure to also take into account and diligently respond to the various other, specific points raised in all three reports. As it is out policy to allow only a single round of revision, acceptance will ultimately depend on the completeness of your responses in this revised version.

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an

extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

Should you have any additional question regarding this decision or your revision, please do not hesitate to contact me. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In the current MS, Youngsoo Lee and colleagues report the effects of ATR deletion during on the embryonic brain. Previous work had already shown that ATR deletion on postmitotic neurons had no obvious impact on the brain size or function (Ruzankina et al Cell Stem Cell 2007). However, reduced levels of ATR during development lead to microcephaly and abnormal brain development (Murga et al 2009). In the context of these works, it was a very sensible idea to perform a careful analysis of how ATR deletion impacts on the embryonic brain. The experiments are very well performed and backed up by good writing and careful analysis. This is definitively a very rigorous and solid analysis of the effects of ATR deletion during brain development, that will be of interest for the scientific community interested on ATR, replication stress and brain development.

My knowledge on embryonic brain development is modest, so that I will restrict myself to comment on the aspects of ATR biology that I am most familiar with, and which I believe could be better clarified. The major claims made here are:

(1) That ATR does not provide a generic protection against replicative stress, but rather a cell-type specific protection. One argument the authors use for this is that there is not a perfect correlation between areas of proliferation and DNA damage accumulation on ATR-KO brains.

Concern: The authors should show images to make exactly this claim. Namely, they should perform a short pulse of BrdU before processing their brains. Then, it would be nice to see a dual gH2AX/BrdU staining that clearly shows that the DNA damage is not most abundant in those regions that proliferate most.

(2) That ATR deletion is distinct from TopBP1 deletion. This data is extremely interesting and unpredicted given that TopBP1 activates ATR.

Concern: However, from looking at the Western Blots one wonders to what extent this is also affected by the different residual amounts of TopBP1 and ATR that still exist on the brains of this mice. The blots show that whereas TopBP1 deletion seems absolute, there is still a faint ATR band. This is important since even small amounts of ATR are still quite proficient, as exemplified on the viability of ATR-Seckel mice. The only way that I can think this could be addressed is by performing a careful comparison of the endogenous levels of phosphorylated Chk1 in several WT, ATR-KO and TopBP1-KO brains.

(3) That, in contrast to what was seen before, p53 deficiency or ATM deficiency do not worsen the phenotypes linked to ATR deletion on the embryonic brain. The authors propose that this could suggest a different role of ATR in preventing genomic integrity in the brain.

Conceptual concern: The reason by which p53 deletion aggravated the amount of replicative stress linked to ATR hypomorphism was proposed to be due to the fact that p53 deficiency could stimulate a more rapid proliferation (Murga 2011). This has been recently also observed to be the case with other mutations that accelerate replication (Gilad et al 2011, Toledo et al 2011). In this context, one potential explanation to these observations is that instead of being related to ATR, this is due to p53 playing a limited role on the replication of neural progenitors. The authors could discuss this possibility.

The same might apply to ATM. Besides the original paper (Murga et al 2009), recent works have also shown that ATR inhibition is also particularly toxic for ATM deficient cells (Reaper PM et al 2011). Hence, rather than this being due to a restricted role of ATR, it is possible that this effect could be due to a restricted role of ATM on these cell types.

Anyway, this is a strong part of their work and I believe could be greatly strengthened by some in vitro work.

Suggested experiment: The authors have nicely provided strong backup for their experiments with the neurosphere-specific deletion of ATR. The authors should use this system to test the effect of p53 or ATM deletion on ATR-KO neurospheres. Whereas I understand that generating new neurospheres for the different mutants might demand a lot of work, the authors could use p53 depleting shRNAs, and ATM inhibitors for this experiments. If these experiments also fail to show any synthetic lethal interaction between p53/ATM and ATR, then this would indicate some substantial hardwiring differences in the way ATR works in mammals.

In summary, I believe that this is an excellent work that will be of interest for the readers of EMBO J, and that could be benefited by these 2-3 small experiments that would help solidify their major claims.

Referee #2 (Remarks to the Author):

This is a broad, though very thorough study of the regional-specific impact of ATR ablation in the developing mouse nervous system. The study employs several very impressive conditional neuronal-restricted knockdown animals and various crosses to characterise the impact of co-ablation of ATR with other genes such as p53 and ATM. The IHC images are very convincing and of a very high quality. This is a good example of how best to do this type of mouse work.

Interestingly, the findings described here differ somewhat from what would have been expected (e.g. not all developing brain regions are equally affected) and from what has been described/assumed for ATR ablation using alternative models (e.g. synthetic lethality of diminished ATR function with p53 or ATM: not reflected here in the developing nervous system). But, in a way herein lies the issue. Whilst this is a beautiful piece of descriptive and comparative analysis, these distinctions are described, but we're none the wiser with regard to why. Timing of the knockdown is of course a very important issue here and the authors have attempted to address this (by using the cre-tm route), but a fundamental issue remains in comparison to say the model described by Murga et al or even Ruzankina et al (i.e. work from one of the groups involved here). Are we just seeing the consequences of differently timed ATR ablation? The latter two involve congenital defects and at face value are in fact more severe than the conditional models described here. This doesn't diminish the authors' findings. This could be relevant for hypomorphic ATR mutations. But it does remain somewhat open-ended. In light of that I wonder if this is in fact more suited to a specialised journal.

Minor comments.

"Microcephaly" is a difficult concept in mice considering its practical usage in clinical genetics (reduced OFC compared to hgt). The ATR(nes-cre) brain is described as microcephalic here (legend Fig 1), but this is really difficult to appreciate given that the animals are in fact very growth restricted (Supp Fig 1). Is this a case of a small brain form a small mouse or is it in fact disproportionally smaller? Taking body weight into account when comparing brain weights would be more appropriate.

The IR-induced DNA damage response work described in Fig 5C doesn't really sit well here in the context of ATR's role during neurodevelopment. Does it tell us anymore than what we already knew from somatic cell work?

Referee #3 (Remarks to the Author):

A hypomorphic mutation in ATR that markedly reduces the protein gives rise to Seckel syndrome and complete knockout of the gene in mice is embryonic lethal. A humanised murine allele (ATRs/s) of Seckels describes the impact of DNA replicative stress on embryogenesis ,resulting in dysmorphic heads and microcephely. Thus this mouse model recapitulated a great deal of the phenotype seen in Seckels. However, it did not address specific effects during brain development. The focus of the present study is on the role of ATR in neurogenesis. Mouse models where Atr is conditionally disrupted throughout the nervous system or only in the dorsal telencephalon are employed. The effect of disruption of Atr in the context of p53 and ATM inactivation is also investigated.
The study is well designed and provides important new information on the impact of DNA replicative stress on neurogenesis. Decreased cellularity is observed in different regions of the brain in response to Atr deletion but there appears to be selectivity for specific progenitor populations. This model assists in our understanding of Seckels as it relates to the role of ATR in development for different tissues.

Specific comments

1. The authors observe severe effects on the cerebellum in AtrNes-cre mice and state that these are due to granule neuron loss, which in turn cause mislocalization of Purkinje cells. Is it not more likely that the defect in Purkinje cells is due to defects in migration of these cells per se?

2. An 80% reduction in proliferation was observed within the AtrNes-cre cerebellar EGL and rhombic lip. In comparison to this proliferative defect they found no increased apoptosis in the embryonic cerebellum between E15.5 - 17". Yet they describe "scattered apoptosis" and gH2AX labelling at E16.5 in the EGL and Rh. This is confusing.

3. Since there is a large effect on proliferation (80% reduction) what is the fate of these cells given that there is only scattered apoptosis?

4. They should specify what part of Fig4 shows the obvious reduction in cortical size.

5. It is of interest that coincident loss of p53 and Atr did not exaserbate the AtrNes-cre phenotype in the nervous system while Ruzankina et al observed regenerative delays in other tissues. The author might provide more insight into this rather than "This may reflect tissue-specificity involving replication stress and p53 signalling".

6. Reference is not made to the Onksen et al Neuropsychopharmacology 36, 960 (2011) article. They show that selective deletion of Atr in the hippocampus of floxed-Atr mice leads to inhibition of new neuron proliferation. The present study reveals reduced cellularity in the hippocampus of AtrEmx1-cre mice. The Onksen data are relevant to this.

7. Minor point page 6 para 2 line 4 ---due to granule neuron (loss ???) leading to ----

1st Revision - authors' response 29 November 2011

Lee et al., "*ATR maintains select progenitors during nervous system development*": Response to Referees.

Referee 1:

The experiments are very well performed and backed up by good writing and careful analysis. This is definitively a very rigorous and solid analysis of the effects of ATR deletion during brain development, that will be of interest for the scientific community interested on ATR, replication stress and brain development.

> We thank the referee for the positive comments. Below we address all points of concern that were raised.

(1) That ATR does not provide a generic protection against replicative stress, but rather a cell-type specific protection. One argument the authors use for this is that there is not a perfect correlation between areas of proliferation and DNA damage accumulation on ATR-KO brains.
Concern: The authors should show images to make exactly this claim. Namely, they should perform a short pulse of BrdU before processing their brains. Then, it would be nice to see a dual gH2AX/BrdU staining that clearly shows that the DNA damage is not most abundant in those regions that proliferate most.

> We have done this and have individually identified BrdU and γH2AX positive cells. We find that the majority of proliferative cells identified by a short pulse of BrdU to be devoid of γH2AX foci. We have also quantified the relative coincidence of proliferation and γH2AX in representative proliferating brain regions. This data is now incorporated into a new figure (Suppl. Fig. 2).
>
> We also note that the cerebellar data in Fig. 2A and Suppl. Fig. 3 also reinforces this concept as these highly proliferative regions show only occasional γH2AX staining. We have now emphasized this point in our text (see page 7), to underscore that most proliferative cells are devoid of γH2AX.

(2) That ATR deletion is distinct from TopBP1 deletion. This data is extremely interesting and unpredicted given that TopBP1 activates ATR.
Concern: However, from looking at the Western Blots one wonders to what extent this is also affected by the different residual amounts of TopBP1 and ATR that still exist in the brains of this mice. The blots show that whereas TopBP1 deletion seems absolute, there is still a faint ATR band. This is important since even small amounts of ATR are still quite proficient, as exemplified on the viability of ATR-Seckel mice. The only way that I can think this could be addressed is by performing a careful comparison of the endogenous levels of phosphorylated Chk1 in several WT, ATR-KO and TopBP1-KO brains.

> We examined pChk1 (ser 317) but the endogenous signal was not readily apparent and not easily discernable from low-level background bands at the position of Chk1. As an alternative, and to also address an issue raised later, we generated multiple neurosphere cultures from $Atr^{Nes\text{-}cre}$ and $Atr^{Nes\text{-}cre};p53^{-/-}$ embryonic

cortical tissue but found that we were unable to generate viable neurosphere cultures surving longer than 7 days when Atr was inactivated. Given the efficiency with which WT neurospheres grow in culture, we interpret this to indicate that efficient deletion of Atr accounts for this lack of neurosphere establishment. The faint Atr signal on western blots from $Atr^{Nes\text{-}cre}$ tissue may also be attributed to blood in the freshly isolated tissue (*Atr* would not be deleted from blood in the $Atr^{Nes\text{-}cre}$ brains). In addition to the developing brain in general, the neocortex has an extensive vasculature associated with cortical progenitor expansion (e.g. Alvarez-Bullya & Lim, Neuron 4, 683-686, 2004).

Additionally, other indices of *Atr* deletion make a compelling case that Atr is inactivated throughout the $Atr^{Nes\text{-}cre}$ (and $Atr^{Emx1\text{-}cre}$) brains. RT-PCR indicates *Atr* is deleted at the genomic level very early in embryogenesis and in all neural tissue examined. The lack of proliferation in astrocyte and neurosphere cultures derived from $Atr^{Nes\text{-}cre}$ tissue also indicates that Atr is deleted. The widespread defects observed in the ganglionic eminence show *Atr* deletion in the ventral telencephalon, while disruption of hippocampal development indicates Atr inactivation in the dorsal telencephalon in $Atr^{Nes\text{-}cre}$ tissue. The lack of Tbr2 staining in the $Atr^{Emx1\text{-}cre}$ hippocampus (Fig. 4E&F) indicates that *Atr* is deleted in very early hippocampal progenitors. The agenesis of the $Atr^{Nes\text{-}cre}$ cerebellum also indicates ATR inactivation.

(3) That, in contrast to what was seen before, p53 deficiency or ATM deficiency do not worsen the phenotypes linked to ATR deletion on the embryonic brain. The authors propose that this could suggest a different role of ATR in preventing genomic integrity in the brain.
Conceptual concern: The reason by which p53 deletion aggravated the amount of replicative stress linked to ATR hypomorphism was proposed to be due to the fact that p53 deficiency could stimulate a more rapid proliferation (Murga 2011). This has been recently also observed to be the case with other mutations that accelerate replication (Gilad et al 2011, Toledo et al 2011). In this context, one potential explanation to these observations is that instead of being related to ATR, this is due to p53 playing a limited role on the replication of neural progenitors. The authors could discuss this possibility.

We have now included a consideration of these points in our discussion; see page 19 (including the above citations). We note that cortical development and brain histology are indistinguishable between WT and $p53^{-/-}$ mice.

The same might apply to ATM. Besides the original paper (Murga et al 2009), recent works have also shown that ATR inhibition is also particularly toxic for ATM deficient cells (Reaper PM et al 2011). Hence, rather than this being due to a restricted role of ATR, it is possible that this effect could be due to a restricted role of ATM on these cell types. Anyway, this is a strong part of their work and I believe could be greatly strengthened by some in vitro work.
Suggested experiment: The authors have nicely provided strong backup for their experiments with the neurosphere-specific deletion of ATR. The authors should use this

system to test the effect of p53 or ATM deletion on ATR-KO neurospheres. Whereas I understand that generating new neurospheres for the different mutants might demand a lot of work, the authors could use p53 depleting shRNAs, and ATM inhibitors for this experiments. If these experiments also fail to show any synthetic lethal interaction between p53/ATM and ATR, then this would indicate some substantial hardwiring differences in the way ATR works in mammals.

To address this we generated neurosphere cultures from multiple E13.5 embryos that resulted in the isolation of 4 individual $Atr^{Nes\text{-}cre}$ and 3 individual $Atr^{Nes\text{-}cre}$;$p53^{-/-}$ embryos (the remaining embryo were various genotypes including *Nes-cre*, and *LoxP*/+). As mentioned above, any neurosphere culture derived from $Atr^{Nes\text{-}cre}$ failed to grow past a week in culture. These neurospheres were clearly distinct from WT or $p53^{-/-}$ neurospheres and although cells were initially present and viable, by 1 week in culture these cells died. Importantly, the initial three $Atr^{Nes\text{-}cre}$;$p53^{-/-}$ cultures were substantially more robust and appeared relatively healthy compared to the $Atr^{Nes\text{-}cre}$ cultures and we were hopeful that these would establish. In that case, we could use KU55933 to determine the outcome of coincident Atr and Atm deletion in an alternative setting to the 4OHT-induced deletion that we show in Fig. 6. However, while the initial $Atr^{Nes\text{-}cre}$;$p53^{-/-}$ cultures looked healthy, both the $Atr^{Nes\text{-}cre}$ and $Atr^{Nes\text{-}cre}$;$p53^{-/-}$ cultures failed to thrive, and all cells eventually died. In contrast, very robust and healthy WT and $p53^{-/-}$ neurospheres were established. From these multiple attempts to generate Atr-null neurospheres we conclude that the chronic lack of Atr is not compatible with cell viability/growth under culture conditions and the additional stresses associated with *in vitro* growth. These data underscore the striking differences between Atr loss *in vivo* and *in vitro*; which we feel is an important finding from our study. We have now added text to this effect to our revised manuscript (page 13, 2nd para) and have included data from the $Atr^{Nes\text{-}cre}$;$p53^{-/-}$ neurosphere cultures (Fig. 6E). We also believe that these culture results reflect the full inactivation of Atr using *Nes-cre* (as would be expected based on the activity of *Nes-cre*) as both WT and $p53^{-/-}$ neurospheres are readily established, and if any progenitors with these genotypes were present we would expect to have these establish in culture during the extended periods we kept these cultures under observation. We do feel that these attempts to generate Atr null neurospheres emphasize the utility of the 4OHT-inducible deletion whereby acute deletion allows characterization of the signaling defects after Atr loss without the absolute requirement for longer-term viability.

Its also noteworthy that the $p53^{-/-}$ mice were germ-line mutants and therefore the lack of exacerbation of the $Atr^{Nes\text{-}cre}$ phenotype in the $Atr^{Nes\text{-}cre}$;$p53^{-/-}$ mice throughout the brain, clearly reflects neural cells deficient for both proteins. We hope this conveys the growth issues associated with Atr-null cells, and that there is a clear enhancement of growth in the Atr/p53 double-null cells initially, although like the single ATR-KO, they also fail to survive longer-term culture.

Referee 2:

This is a broad, though very thorough study of the regional-specific impact of ATR ablation in the developing mouse nervous system. The study employs several very impressive conditional neuronal-restricted knockdown animals and various crosses to characterise the impact of co-ablation of ATR with other genes such as p53 and ATM. The IHC images are very convincing and of a very high quality. This is a good example of how best to do this type of mouse work.

Thank you very much for the positive comments.

Interestingly, the findings described here differ somewhat from what would have been expected (e.g. not all developing brain regions are equally affected) and from what has been described/assumed for ATR ablation using alternative models (e.g. synthetic lethality of diminished ATR function with p53 or ATM: not reflected here in the developing nervous system). But, in a way herein lies the issue. Whilst this is a beautiful piece of descriptive and comparative analysis, these distinctions are described, but we're none the wiser with regard to why. Timing of the knockdown is of course a very important issue here and the authors have attempted to address this (by using the cre-tm route), but a fundamental issue remains in comparison to say the model described by Murga et al or even Ruzankina et al (i.e. work from one of the groups involved here). Are we just seeing the consequences of differently timed ATR ablation? The latter two involve congenital defects and at face value are in fact more severe than the conditional models described here. This doesn't diminish the authors' findings. This could be relevant for hypomorphic ATR mutations.

We take the referee's point and agree that there are molecular details regarding cell-type programming that impact ATR functions. A notable point, and one which is illustrated by the *TopBP1*$^{Nes\text{-}cre}$ data, is the mild cortical effects of Atr inactivation found in *Atr*$^{Nes\text{-}cre}$ mice. In comparison to the work of *Murga et al* and *Ruzankina et al* in which Atr function was examined using germ-line Atr mutations, or inducible inactivation, our data reveal key features of tissue-specificity and deletion timing. Our study expands and refines these concepts to show that genetic interactions between ATR and p53 loss are context dependent.

Minor comments.

"Microcephaly" is a difficult concept in mice considering its practical usage in clinical genetics (reduced OFC compared to hgt). The ATR(nes-cre) brain is described as microcephalic here (legend Fig 1), but this is really difficult to appreciate given that the animals are in fact very growth restricted (Supp Fig 1). Is this a case of a small brain from a small mouse or is it in fact disproportionally smaller? Taking body weight into account when comparing brain weights would be more appropriate.

We appreciate that the usage of the word microcephaly is unclear. Although we followed the convention of reduced head circumference (3 standard deviations below the mean) and because ATR-Seckel syndrome is related to primordial dwarfism syndromes, we adopted the microcephaly term. However, to avoid

interpretational issues we have amended our language to read small brain and body size (see page 6, first section of results), and have also added a new figure (Suppl. Fig. 8) detailing body and brain sizes in the Atr/p53 mutants to convey a clearer account of our data.

The IR-induced DNA damage response work described in Fig 5C doesn't really sit well here in the context of ATR's role during neurodevelopment. Does it tell us anymore than what we already knew from somatic cell work?

We think this analysis is relevant because of our focus on the nervous system and that there is no reported *in vitro* ATR studies using this system. We also considered this useful data because of the disparity in a mild perturbation in neural development compared to substantial effects toward Atr-null astrocyte growth and DNA damage accumulation. Additionally, the neurospheres reflect a closer scenario to Atr signaling in progenitors in the brain than other ATR-depleted cell types. These data also complement new data incorporated into Fig. 6 in which we investigate the effect of coincident p53 and Atr deletion, and provide additional support that Atr deficiency in cells expressing a bona fide neural transcriptional program tolerate dual inactivation, where p53 loss partially rescues Atr loss (at least in the short term)

Referee 3

The study is well designed and provides important new information on the impact of DNA replicative stress on neurogenesis. Decreased cellularity is observed in different regions of the brain in response to Atr deletion but there appears to be selectivity for specific progenitor populations. This model assists in our understanding of Seckels as it relates to the role of ATR in development for different tissues.

Thank you, we appreciate the positive comments.

Specific comments

1. The authors observe severe effects on the cerebellum in AtrNes-cre mice and state that these are due to granule neuron loss, which in turn cause mislocalization of Purkinje cells. Is it not more likely that the defect in Purkinje cells is due to defects in migration of these cells per se?

We based this on other studies in which granule cell loss leads to a failure of Purkinje cells localization, as the enormous postnatal expansion of granule cells in the inner cerebellar region (migration from the outer germinal zone) generates the physical aspects of the cerebellum that serves to align the Purkinje cells. This is consistent with reports in the literature on cerebellar development in which specific granule neuron ablation from various sources results in the loss of cerebellar lobulation and subsequent Purkinje cell alignment (e.g. Doughty et al., J. Comp. Neurol. 399, 306-320, 1998).

2. An 80% reduction in proliferation was observed within the AtrNes-cre cerebellar EGL and rhombic lip. In comparison to this proliferative defect they found no increased apoptosis in the embryonic cerebellum between E15.5 - 17". Yet they describe "scattered apoptosis" and gH2AX labelling at E16.5 in the EGL and Rh. This is confusing.

> We have modified the text to more accurately indicate our findings (page 7). Our modified language now reads that a small level of apoptosis was observed, but that this is sporadic and not the cause of the failed granule neuron expansion. Our intent was to indicate that the defects in cerebellar development are related to a failure of the EGL to proliferate; we also included details in the discussion suggesting that this was likely related to the rapid expansion and associated replication stress that this structure undergoes in response to sonic hedgehog signaling.

3. Since there is a large effect on proliferation (80% reduction) what is the fate of these cells given that there is only scattered apoptosis?

> We have not carefully examined the fate of these cells. However, based on morphology/histology, we found nothing obvious in the EGL to suggest a degenerative process was occurring. The EGL shows normal morphology, but does not undergo proliferation and expansion in a manner like the WT counterpart.

4. They should specify what part of Fig4 shows the obvious reduction in cortical size.

> We have now referred to Figure 4 panel E (page 9).

5. It is of interest that coincident loss of p53 and Atr did not exacerbate the AtrNes-cre phenotype in the nervous system while Ruzankina et al observed regenerative delays in other tissues. The author might provide more insight into this rather than "This may reflect tissue-specificity involving replication stress and p53 signalling".

> We were being conservative in our speculation, but have now expanded this statement to contain more substance, and have used the different outcomes of ATM loss in the nervous system compared with other tissues to support the tissue-specificity suggestion. (see Discussion, Page 19)

6. Reference is not made to the Onksen et al Neuropsychopharmacology 36, 960 (2011) article. They show that selective deletion of Atr in the hippocampus of floxed-Atr mice leads to inhibition of new neuron proliferation. The present study reveals reduced cellularity in the hippocampus of AtrEmx1-cre mice. The Onksen data are relevant to this.

> The Onksen reference has now been cited (page 5).

7. Minor point page 6 para 2 line 4 ---due to granule neuron (loss ???) leading to --

> We have corrected this grammatical error.

Acceptance letter 14 December 2011

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Before we will able to send you a formal letter of acceptance, I need to ask you for one minor thing, a 'conflict of interest' statement to be included at the end of the manuscript text. You may simply send them to us in the body of an email, from which we can easily copy them into the manuscript text file. Furthermore, please let me know if you would still want to make some minor modifications to the manuscript text in light of the remaining comments of the referees - in this case please simply send us a modified text file (including such changes and the conflict of interest statement) as email attachment.

After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Yours sincerely,
Editor
The EMBO Journal

Referee #1

(Remarks to the Author)
The authors have made an important effort in responding to all of the referees comments, and I believe the MS is now a very good contribution to be published at the EMBO Journal.

I just want to take this opportunity to make two smal notes to the authors:

It is a pitty that the essential nature of ATR in the neurosphere experiments did not allow for the testing of ATM and p53 interactions with ATR in this system, which would have helped in the mechanistic understanding of the phenotypes.

In what regards to the TopBP1/ATR difference, I still am not 100% sure that this is not due to differences in residual amounts of ATR (vs TopBP1) in this system. But I understand the points made by the authors, and I also can see that the analysis of the TopBP1 strain demands a further independent work. It would have been very interesting to see whether, in contrast to ATR, TopBP1 deletion is compatible in neurospheres.

Referee #2

(Remarks to the Author)
The authors have done an admirable job in addressing the original referee comments and have included extra experimental work to justify their arguments. I have a few relatively minor comments and suggestions the authors may wish to consider.

i). When discussing the functional overlap between ATM and ATR (p14-16 & discussion), the authors could make reference to the fact that what is utterly clear is that loss/reduced function of each kinase results in clinically distinct manefestations in humans (A-T: a progressive neurodegeneration versus Seckel syndrome: a microcephalic dwarfism). The different neuropathologies reinforce the authors arguments that "Atr and Atn function independently in the developing nervious system..."). This could be brought-out in the manuscript.

ii). Perhaps a summary model figure, contrasting the finding here to what has been shown for

somatic cells (relationships between ATR, ATM, p53) along with a schematic summarising the brain regions predominantly and selectively affected following loss of ATR 'v' TopBP1 'v' ATM, would be helpful to strengthen the 'take- home' message.

iii). Discussion section, "Our data showed..." Point 3: I think it somewhat an overstatement to claim that the data here shows "that ATR-Seckel syndrome manefests a complex neural phenotype...". It's more accurate to claim that loss of Atr shows this, based on the data presented. After all, the humanised Atr mouse bearing the Seckel syndrome mutation was not investigated here and we know from this work and cellular analysis that the ATR-Seckel mutation described is highly hypomorphic. This would be expected to modify the very interesting brain phenotypes described here, based on Atr loss, when impaired ATR function is seen in a viable human.

iv). Similarly, I think the final statement in the last line of the discussion ("...alleviating the spectrum of pathology in human DNA repair deficiency syndromes") is probably a bit of an over-interpretation. In fairness, the consequences of reduced ATR function occur during development and are fixed in the postnatal context. Therefore, since most treatments are not directed towards the developing embryo, it's probably best to just cut that bit of the sentence out!

Additional correspondence (author) 15 December 2011

Thank you very much for the great news! We are very excited to see this work published in the EMBO Journal.

In addition to a conflict of interest statement, I would like to incorporate the minor text changes suggested by Reviewer #2 and therefore will send you a revised text doc with in a day or so.

